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14. ABSTRACT

There is both considerable interest and ignorance in the possible role of iodine in the etiology and prognosis of breast cancer. This project is the first step in elucidating a mechanistic role for iodine in breast carcinogenesis. The data that we have been able to generate to date suggest that our hypothesis is correct; namely, using transgenic human breast cancer cells (MCF7) overexpressing the sodium/iodide symporter (NIS) and/or lactoperoxidase (LPO), we have shown that NIS facilitates death or survival pathways following irradiation, a known human breast carcinogen, depending on the presence or absence of iodine, respectively, and that this switching can be modulated by the cell's ability to organify and stabilize the iodine via LPO. Further, we have shown that expression of both NIS and LPO will radiosensitize the MCF7 cells while NIS alone will make them radioresistant and more aggressive. These data agree with observations made by others demonstrating that iodine deficiency is correlated with increased breast cancer incidence, and that a large percentage of human breast cancers overexpress NIS. Additionally, the fact that NIS and LPO are most active in the mammary glands during late pregnancy and lactation may explain the well established observation that early and frequent parity and long lactation history reduce the risk for breast cancer development. We are confident that the data from the experiments currently in progress should help to strengthen our already existing results. Clarification of these issues should foster future studies not only in breast cancer diagnosis and therapy but also in prevention through conscious changes in diet and environment.

15. SUBJECT TERMS
breast carcinogenesis, iodine, NIS, LPO, ionizing radiation

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INTRODUCTION

Dietary iodine intake is generally associated with proper thyroid function. There is an estimated 30-50 mg of iodine in the human body; however there is less than 30% present in the thyroid gland and its hormones, namely T_3 and T_4. (1) The remaining approximately 70% is non-hormonal and is concentrated in extra-thyroidal tissues; that is, in the salivary glands, the gastric mucosa, and the mammary glands. (2) Iodide is required for all plant and animal cells but, except for its nearly proverbial role in iodinated hormones in vertebrates, in large part the biological role of this essential element is unknown. Herein lies an unsettled controversy that has smoldered for decades over a possible association between thyroid disease and breast cancer.

A large volume of epidemiological studies correlate thyroid disease with breast cancer. Both diseases are female dominant with similar postmenopausal peak incidence. Associations have been found between breast cancer and hypothyroidism, T_4-replacement therapy, hyperthyroidism, thyroiditis, and endemic goiter. (3) For example, studies show that the incidence of breast cancer is low in areas where endemic goiter is rare, whereas the reverse is true where thyroid diseases are quite common. (4) Laboratory studies have also supported the connection between thyroid disease and breast cancer. For example, animal experiments have demonstrated that an iodine-rich diet reduces carcinogen-induced mammary tumorigenesis, supporting population studies that show a low incidence of breast cancer in women who consume an iodine-rich diet. (5) Further it is worth noting that the iodine absorption in the breast occurs in the same ductal epithelium where the majority of breast cancers arise suggesting that disruption of iodine homeostasis, such as through iodine deficiency, could lead to promotion of breast carcinogenesis. (1, 6, 7, 8)

Because iodine is environmentally scarce, animals including humans have evolved a system of concentrating it in certain organs. The major mode of iodine transport into the cell is via the sodium/iodide symporter (NIS). NIS is an integral plasma membrane glycoprotein that mediates active transport of iodine by coupling the inward 'uphill' translocation of I^- to the inward 'downhill' translocation of Na^+ generated by the sodium/potassium pump. (9, 10, 11) NIS was once believed to be a thyroid-specific protein; however, it is now clear that NIS is also expressed in the salivary glands, the gastric mucosa, and the mammary glands. (2) The outstanding feature of the mammary gland is that whereas iodine transport is constitutive in the other tissues, it occurs exclusively during late pregnancy and lactation in the mammary gland. (12) The physiological significance of the high uptake of iodine by the mammary gland has been explained as a vital evolutionary mechanism to provide the neonate with a supply of iodine for proper neural development via T_3 and T_4 hormonal control. (13, 14)

To fully take advantage of the NIS-associated cellular influx, the iodine must be organified; that is, the atoms must be covalently bound to intracellular proteins or lipids. In the thyroid, iodide acts as an electron donor in the presence of H_2O_2 and thyroperoxidase and the remaining iodine atom readily iodinates tyrosine, histidine, or certain specific lipids. (15) Similarly in the mammary gland, lactoperoxidase (LPO) catalyzes the reaction. LPO, a monomeric glycoprotein, is one of the most prominent enzymes in milk and is particularly active during pregnancy and lactation. (1, 16, 17)

Therefore, LPO and NIS are the two key enzymes in iodine metabolism in the mammary gland. The essential facts relating iodine and breast cancer together with epidemiological and laboratory data begin to weave an interesting testable hypothesis on the mechanistic origins of the disease. In recapitulation, 1) iodine intake is associated with decreased risk for breast cancer, 2) NIS and LPO are expressed in the mammary glands only during late pregnancy and lactation, and 3) parity and long lactation history decrease the risk for breast cancer. Additionally with respect specifically to breast cancers, LPO is seldom expressed in human breast cancer cell lines and NIS is over-expressed in an unprecedented 80-90% of human breast cancers. (12) The mechanism of *NIS* gene-induction in the breast is unclear. Clearly, however, NIS must confer a survival advantage (or be critically linked with something that does) to explain the high frequency of NIS-expressing breast cancers. Yet these observations conflict with the breast cancer-inhibiting effect of iodine and the decreased risk for breast cancer development associated with early and frequent parity (and lactation) that result in high expression of NIS. There are zero reports in the literature studying/modeling the mechanistic role of NIS expression in breast carcinogenesis.

To address these questions, we investigated the role of NIS, LPO, and iodine in breast carcinogenesis using an ionizing radiation (IR) tumorigenesis model. IR is an exemplary breast carcinogen because it is well characterized in terms of risk and dose (primarily from A-bomb survivor data) and because primary exposure to it is frequent (from medical x-rays) but can be controlled if warranted. Risk for tumorigenesis of breast is the highest (along with thyroid, interestingly) among IR-attributable solid cancers. Early age at first full-term pregnancy, multiple births, and lengthy total lactation history are all protective against IR-related breast cancer. (18) Such life events may induce terminal differentiation and/or facilitate apoptotic deletion of preneoplastic cells. The reasons are unresolved. We hypothesized that mammary NIS facilitates pro- or anti-apoptotic pathways following irradiation depending on the presence or absence of I^-, respectively, and that this switching can be modulated hormonally. A study has shown that induction of both NIS and thyroperoxidase was necessary to elicit apoptosis by I^- in lung cancer cells. (19) Hence, we further hypothesized that normal lactating mammary cells express both NIS and LPO, conferring protection against tumorigenesis, whereas breast cancer cells express *only* NIS, conferring a survival advantage. The testable corollary, then, is that transgenic expression of both NIS and LPO in a breast cell line will radiosensitize it while NIS alone will make it radioresistant and tumorigenic (or more aggressive if already tumorigenic).

METHODS, MAJOR SETBACKS AND PROGRESS

The cDNAs for both hLPO and hNIS were unobtainable from the anticipated sources. This led to a setback of 5-6 months as we had to clone the genes ourselves from scratch. Both genes were successfully cloned from a human cDNA library and ligated for eukaryotic expression into pIRESpuro3 or pIRESneo3 vectors (BD Biosciences, Clontech, Palo Alto, CA), respectively. MCF7 human breast cancer cells were liposomally transfected first with pIRESneo3-hNIS or empty vector and cloned through selection in G418. Positive clones were then used for transfection with pIRESpuro3-hLPO or empty vector and cloned through selection in puromycin. This series led to the creation of four cell lines: MCF7(-NIS/-LPO), MCF7(+NIS/+LPO), MCF7(-NIS/+LPO), and MCF7(+NIS/-LPO). MCF10A human normal breast cells were similarly treated. Final cloning is still in progress.

The four cell lines described above, MCF7(-NIS/-LPO), MCF7(+NIS/+LPO), MCF7(-NIS/+LPO), and MCF7(+NIS/-LPO), were used to assess growth, clonogenic survival, and invasiveness following a series of various dose treatments with ionizing radiation and/or iodine concentration. Some of the experiments are still in progress. Figure 1 shows the results of 3 independent clonogenic survival assays. Figure 2 shows the results of 3 independent experiments on cloning efficiency as a measure of the effects of iodine intake and organification on the viability of the cell lines. Figure 3 shows the results of 3 independent experiments on the effect of iodine intake and organification on growth of the cells lines before and after irradiation. Figure 4 shows the alterations in invasiveness of the cells caused by expression of NIS and/or LPO. The in vivo experiments of tumor growth of the cells lines in mice are currently in progress.

RESULTS AND DISCUSSION

There is seemingly contradictory data in the literature regarding the role of iodine in breast carcinogenesis. On the one hand, iodine deficiency increases the risk for development of breast cancer but on the other hand NIS is over-expressed in a majority of breast cancers suggesting that increased ability to uptake iodine increases the survival advantage of breast cancer cells. Our results to date offer a possible explanation for this paradox. Although the data generation for the non-tumorigenic cell lines is currently in progress, the results from the human cancer cell line MCF7 contribute to elucidating the solution.

Ionizing radiation is a well-established human breast carcinogen. Using a radiation carcinogenesis model, we show in Figure 1 that over-expression of NIS confers radioresistance to MCF7 cells. This effect is confirmed to be due to the intake of iodine since addition of perchlorate, a strong competitive inhibitor of iodine, abrogates the gained radioresistance. These results help to explain the high frequency of breast cancer that over-express NIS because they suggest that high intake of iodine increases resistance to radiation-induced death. However these results still do not explain the risk reduction effect of iodine demonstrated in epidemiological studies.

The mechanistic explanation for these observations may be found in Figure 2, which shows plating efficiency decreases when LPO is over-expressed, suggesting that in order for iodine to effect decreased survival of breast cancer cells, it must enter the cell *and* be organified. Further, Figure 2 shows that over-expression of NIS alone without LPO, also reduces plating efficiency although to a lesser degree than when LPO is co-expressed; again, the addition of perchlorate verifies that the effect is due to iodine intake. Thus the data in Figure 2 complements that in Figure 1 by demonstrating that although iodine intake confers *relative* radioresistance, the absolute number of surviving cells is greatly reduced when LPO is co-expressed.

Moreover, Figure 3 illustrates the distinct behaviors of the apparently equally radioresistant cells that express only NIS and that express both NIS and LPO. When LPO is co-expressed, the data show that the surviving cells' growth rate after irradiation is below control, but when only NIS is expressed, the growth rate is significantly above control cells and unirradiated cells. Additionally, as shown in Figure 4, expression of LPO with or without NIS expression decreases the invasiveness of these cells, but expression of NIS alone enhances it implying that a +NIS/-LPO phenotype increases the aggressive nature of the cancer cells. The effect is confirmed to be due to iodine because addition of perchlorate abrogates the invasive behavior. These results suggest that our hypothesis is correct in that radiation-induced breast carcinogenesis is enhanced by NIS expression but is minimized by NIS and LPO expression. Further confirmation await the results of the non-tumorigenic breast cell line and the in vivo mice results currently in progress.

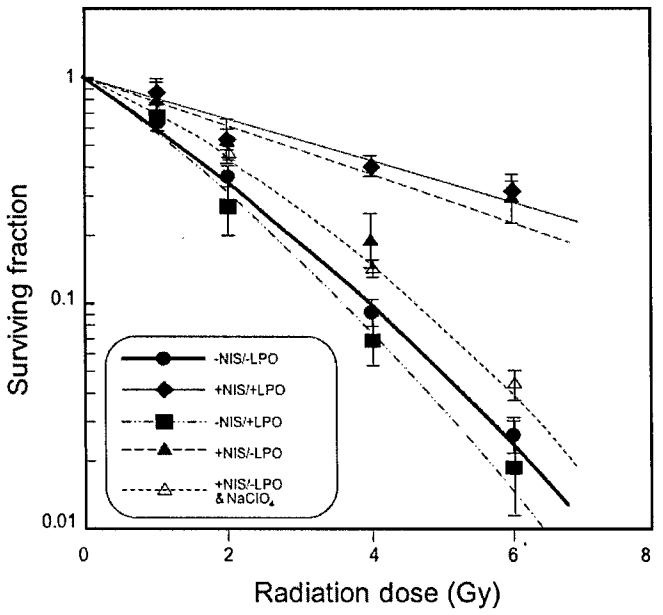

Figure1. Clonogenic survival of cells following irradiation. Cells were irradiated at various doses and plated into dishes with complete medium containing 1mM KI or 1mM $NaClO_4$ where indicated. The colonies were allowed to grow for 21 days after which the cells were fixed, stained, and enumerated.

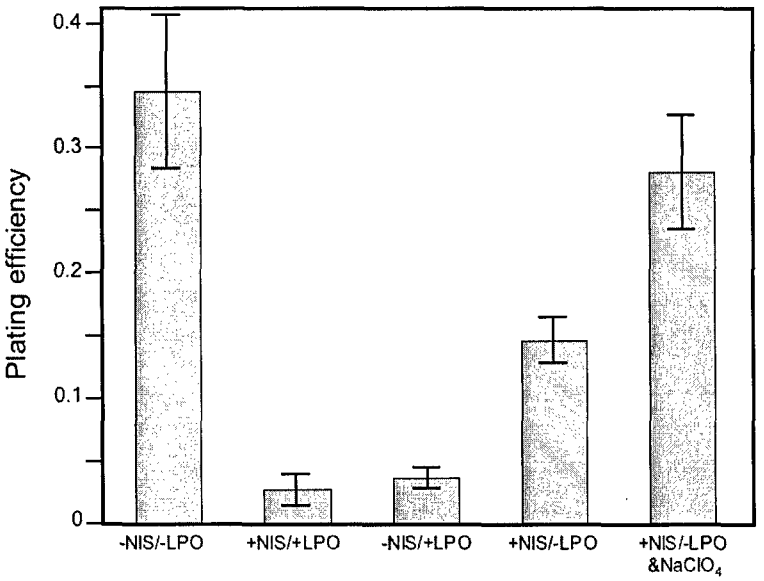

Figure 2. Plating efficiency of cells. Known numbers of cells were plated in medium containing 1mM KI or 1mM $NaClO_4$ where indicated. The cells were allowed to grow for 21 days and the colonies were fixed, stained and enumerated. The plating efficiency was calculated as the ratio of counted colonies to number of cells originally plated.

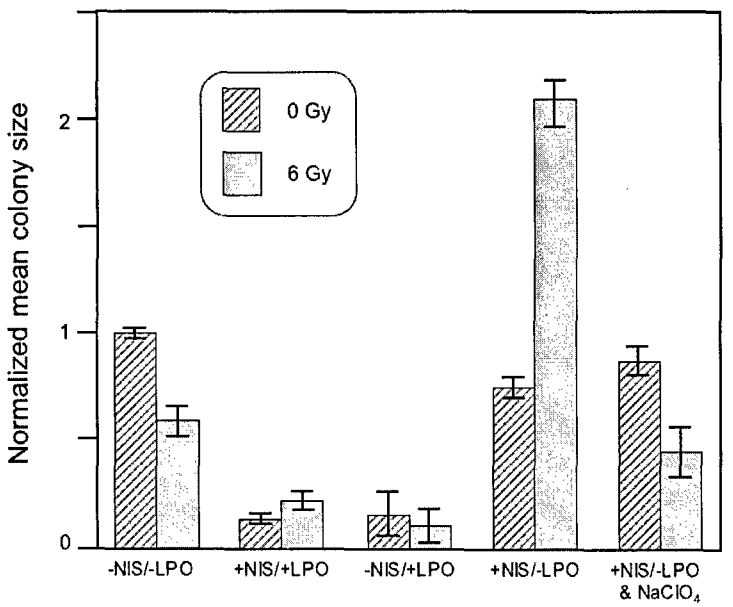

Figure 3. Growth potential of cells. Cells were mock irradiated or irradiated with 6 Gy of Cs-137 gamma-rays and plated in medium containing 1mM KI or 1mM $NaClO_4$ where indicated. The cells were allowed to grow for 21 days after which they were fixed and stained. The dishes were scanned and digitized. The gray level threshold for colonies were determined and the total number of pixels above this threshold was divided by the total number of colonies to give the average pixels per colony which was then normalized to the control.

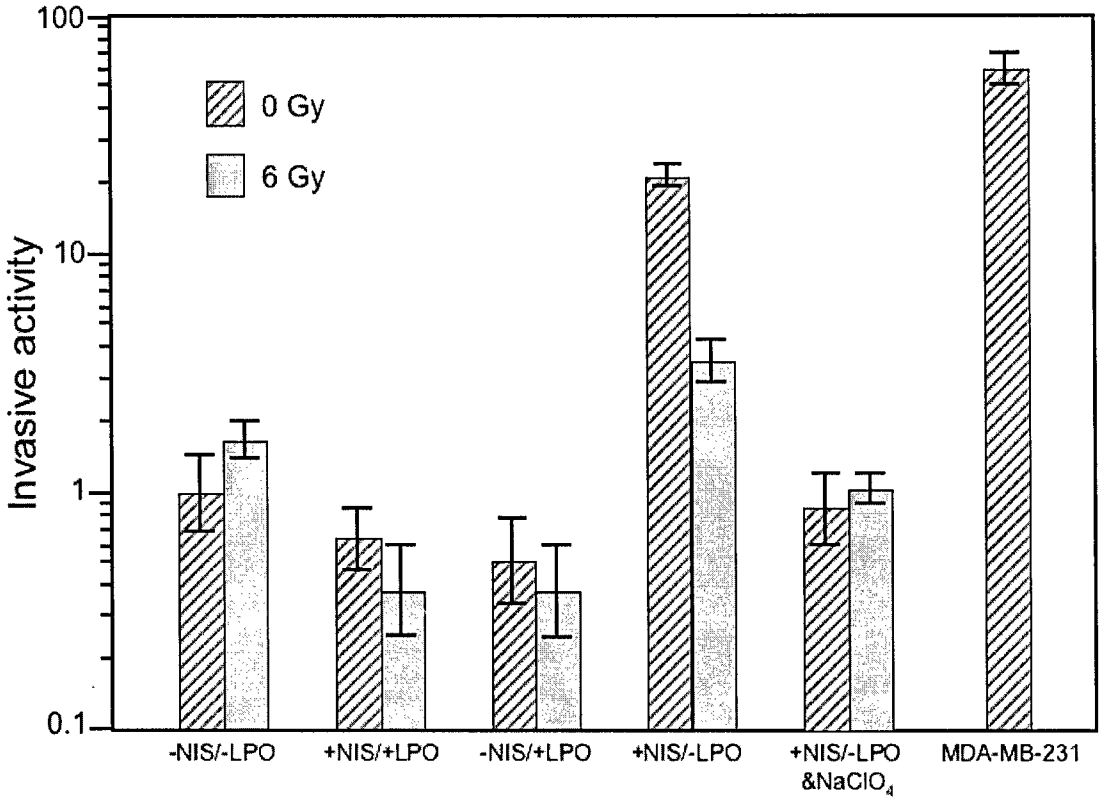

Figure 4. Invasive activity of cells. Cells were mock irradiated or irradiated with 6 Gy of Cs-137 gamma-rays and plated in medium containing 1mM KI or 1mM $NaClO_4$ where indicated. After 5 days of growth/recovery, the cells were trypsinized and 50,000 viable cells (167,000 cells/cm^2), as assessed by trypan blue dye exclusion, were plated in 8-micron pore Boyden's chamber inserts coated with matrigel (BD Biosciences, Bedford,MA) in a serum-less medium. The inserts were placed in wells containing complete medium enriched with 100ng/ml prolactin and 10ng/ml EGF. The setup was incubated at 37C for 36 hours. The cells that had invaded the matrigel and membrane were fixed, stained and enumerated. MDA-MB-23, known to possess an invasive phenotype, was used as a positive control. Note that the scale is logarithmic.

KEY RESEARCH ACCOMPLISHMENTS

- Cloning of hNIS
- Cloning of hLPO
- Establishment of MCF7 cells transfected with hNIS and/or hLPO
- Mechanistic substantiation of the critical role of iodine in breast carcinogenesis by demonstration of:
    - conferral of radioresistance as a result of hNIS expression
    - enhanced growth post-irradiation as a result of hNIS expression
    - decreased growth post-irradiation as a result of both hNIS and hLPO expression
    - enhanced invasiveness as a result of hNIS expression
    - decreased invasiveness as a result of both hNIS and hLPO expression
    - enhanced survival as a result of hNIS expression
    - decreased survival as a result of both hNIS and hLPO expression

REPORTABLE OUTCOMES

- Abstract for Era of Hope Breast Cancer Conference, Philadelphia 2005
- Anticipated manuscript reporting the mechanistic importance of iodine in breast carcinogenesis
- Application for funding from the American Institute of Cancer Research
- Anticipated breast carcinogenesis animal model

PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT

Keisuke S. Iwamoto	Principal Investigator
Kwang-hee Kim	Staff Research Associate

CONCLUSIONS

There is both considerable interest and ignorance in the possible role of iodine in the etiology and prognosis of breast cancer. This project is the first step in elucidating a mechanistic role for iodine in breast carcinogenesis and in explaining the unprecedented observation that 80% of breast cancers overexpress NIS. Although we are still in the progress of accomplishing everything stated in our Statement of Purpose, the data that we have been able to generate to date suggest that our hypothesis is correct; namely, that both NIS and LPO are critical in breast carcinogenesis. Specifically, the organification by LPO of the iodine transported by NIS is important in decreasing the aggressive nature of breast cancer cells but that importation of iodine

via NIS without organification by LPO leads to an enhanced growth of the breast cancer cells. These data agree with observations made by others demonstrating that iodine deficiency is correlated with increased breast cancer incidence, and that a large percentage of human breast cancers overexpress NIS. Additionally, the fact that NIS and LPO are most active in the mammary glands during late pregnancy and lactation may help to explain the well established observation that early and frequent parity and long lactation history reduce the risk for breast cancer development. We are confident that the data from the experiments currently in progress should help to strengthen our already existing results. Clarification of these issues should foster future studies not only in breast cancer diagnosis and therapy but also in prevention through conscious changes in diet and environment.

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